#19
B. Webb
1/24/02

DKT. 13131

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Susan L. Weston *et al.*	Art Unit:	1655
Serial No.:	09/228,639	Examiner:	Enewold, J.
Date Filed:	12 January 1999	Docket No.:	13131
For:	SEQUENCES		

January 21, 2002

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

DECLARATION

Sir:

1. I, Dr. Gary Brown, am skilled in the art of Molecular Biology, and I am employed at Orchid Biosciences. I have a Ph. D. in Molecular Biology. Attached as Exhibit A is my Curriculum Vitae.

2. This declaration under 37 CFR 1.132 is made in support of the Preliminary Amendment. I make this declaration based upon my training, knowledge, education, and experience as a molecular biologist, my review of the application and the history of the prosecution of this application as reflected in the file maintained by the attorneys prosecuting this application, and my review of prior art cited by the Examiner during prosecution of this application, and my review of inventor laboratory monthly project reports (laboratory reports) as cited below.

3. I have reviewed the October 13, 2000 Office Action in the above-captioned case

including the portion on pages 8-9 where the Examiner states that the arguments of counsel cannot take the place of evidence in the record. The Examiner is referring to Applicants' September 14, 2000 response. The Examiner states that attorney statements are not evidence, and statements regarding unexpected results (of multiplexing certain primers), must be supported by an appropriate declaration. In the following, I address the unexpected results of the present invention.

4. I have reviewed the laboratory reports of an inventor Susan Weston (Susie Weston), two of the reports are attached as Exhibits B and C. As one skilled in the art, I conclude that these laboratory reports detail the unexpected results. The reports record difficulties, the multiple failures, and the surprising results of the multiple experiments performed by the inventor in the present application. These experiments show unexpected results after modifications of numerous parameters of the experimental system.

5. Inventor Susie Weston, in her laboratory report from Lab books NBY 5882 and NBY 5922, shows that certain primers were prone to non-specific priming which makes such primers unacceptable for use in this assay. A number of unacceptable primer dimer results were observed, and a reversed direction primer was selected and introduced as a standard, to avoid primer dimers (Exhibit B, page 1). In addition, zinc concentrations and temperature were varied. Some of the unacceptable results the inventor documented were weak signal, very weak signal, primer dimer, and nonspecific bands (Exhibit B, page 2). In further experiments, the inventor designed new primers, varied concentrations of primers, tried zinc-free preparations, and varied the annealing times. Results of these modifications that the inventor recorded

included unacceptably faint signals and smeared products, absent product, and nonspecific products (Exhibit B, page 3). I conclude that the inventor designed multiple variations of the experiment expecting to overcome problems of nonspecificity, weak products, and primer dimer contaminants, but she unexpectedly found the results of absent products, and some of the same problems. This necessitated further variations in further experiments.

6. Inventor Susie Weston, in her laboratory report from Lab books NBY 5922 and NBY 5935 (Exhibit C) concludes that the results she obtained from further experiments were unexpected and were not routine. Following experiments that attempted to eliminate nonspecific products and increase band intensities—problems from the previous months' results—some resulting signals were again too weak, and so she varied the concentrations of primers. She also varied primer length, using 29mers, 26mers, 24mers, and also temperature changes in the protocol as well (Exhibit C, page 1). I particularly note the following observation by her: "This **surprisingly had the reverse effect -all ...DNAs were specific with the original primer and non-specific with the new primer"** (Exhibit C, page 1, emphasis added). She went on to vary concentration of primers, lengths of primer, dropped certain primers, varied annealing temperatures, varied Taq enzyme units, and varied DNA concentrations (Exhibit C, page 2). Results she described were weak, very weak, nonspecific—i.e. unacceptable.

7. Inventor Susie Weston, in her laboratory report from Lab books NBY 5935 and NBY 6107, shows her experimental variation of dNTP reagent concentration, and design of new primers to attempt to overcome mispriming difficulties. Primer length and

identity of primers were varied. Her results included nonspecific bands, weak diagnostics, and extremely weak non-specific bands (Exhibit C, page 3). A result noted by the inventor states **"[i]t was thought it might come up 621+1 as only the 3' base would be mismatched but much to our relief it didn't."** (Exhibit C, page 3, paragraph 3). Nonspecific signals and weak signals continued to be problematic, which shows that even more experimentation was needed. Accordingly, I conclude that the inventors modifications sometimes resulted in improved assays other times resulted in new or continued problems, and that these results to obtained the presently disclosed primers were not routine.

8. I have reviewed Little et al. EPO 497527A1 (Little); Ferrie et al. Am. J. Human Genetic, 51:251-262 (1992) (Ferrie); Estivil et al. Human Mutation, 10-135-154 (1997) (Estivil); Cystic Fibrosis Genetic Analysis Consortium, Human Mutation, 4:167-177 (1994) (CFGAC) cited by the Examiner. I conclude that there is no teaching or suggestion in any of these references, alone or in combination, of the specific primers described in the present patent application, such as for example, having the lengths, concentrations and combinations. Further, I conclude there is no teaching or suggestion in any of these references, taken alone or in combination, disclosing the methods of using these primers in multiplex analysis with the unexpected results shown in the present patent application.

9. With regards to the Ferrie reference cited by the Examiner, Ferrie discloses the difficulty of multiplexing primers directed to just four mutations. The present invention involves the unexpectedly successful multiplexing of up to twelve primers. Thus, the inventions' primers are unexpectedly able to detect specific CFTR

mutations reliably and robustly in a multiplexed mixture.

10. I have further reviewed the portion on page 8 of the October 13, 2001 Office Action in the above captioned case where the Examiner states as follows:

> ....in response to applicant's arguments that multiplexing of more than four primer sets is taught away from based upon the practical limits, Schumm et al. (US Pat. 6,843,660), December 1998) teaches the multiplexing of eight STR loci (example number 19). Further, Schumm teaches that successful combinations can be generated by trial and error of locus combinations, by selection of primer pair sequences, and by adjustment of primer concentrations to identify an equilibrium in which all included loci may be amplified. Thus the art has taught the multiplexing of eight different loci in a single reaction vessel and thus, six primer sets of the instant invention is well within the realm of practical limits.

11. I disagree with the Examiner's position and maintain that one of ordinary skill in the field of molecular biology would ***not*** be motivated to combine this reference with the others cited (Estivill, Ferrie, Little, and CFGAC). I have carefully reviewed the Schumm reference relied on by the Examiner, and have concluded that its focus is Short Tandem Repeat (STR) loci, which are different from the subject matter of the present invention, which involve mutations in the CFTR gene. STR loci are regions of the genome that contain short, repetitive sequence elements of 3 to 7 base pairs in length. Schumm et al. U.S. Patent 6,843,660 notes that polymorphic STR loci are extremely useful markers for human identification, paternity testing and genetic mapping. Schumm states that while there are multiplex amplification procedures for specific loci, the use of multiplex amplification procedures is greatly desired for the

detection of alleles in other types of loci such as specific STR loci and that it is also desirable to identify primers which make multiplex amplification of such loci possible. Schumm merely cites Ferrie as a general reference discussing multiplex amplification sets for analysis of genes related to human genetic diseases. Since Schumm distinguishes STR loci from those related to human genetic disease, one of ordinary skill in the art would not be motivated to combine Schumm with Ferrie or any of the other cited references in order to arrive at the present invention.

12. Further, Ferrie actually reveals a long-felt need: "clearly, a system which could simultaneously analyze a sample for the presence of multiple mutations would be useful." Page 252. "It is clear, however, that, for many inherited and acquired mutations, simultaneous analysis of several mutations is desirable." Page 260. Moreover, failure of existing technology to meet this long-felt need is stated in this same reference: "Initially, the primer sequences selected for the single ARMS tests were combined into multiplex reactions. This approach did not work, because the yields of several of the reactions were too low...or too high...when compared with the yield of the other reactions. Page 260.

13. Schumm is cited by the Examiner as disclosing multiplexing of 8 STRs and the Examiner states the reference teaches multiplexing. I disagree. This reference instead expresses a long-felt need for the multiplexing of the present invention. The disclosure of Schumm is different from the present invention. The STRs disclosed in Schumm are markers, not mutations. Markers are generally not mutations. The STR analysis, as disclosed in Schumm, provides a measure of genetic diversity without determining the identity of any disease-causing mutation. This differs from the present invention, which identifies specific mutations in genes, where the mutations cause a severe disease, cystic fibrosis. The information about which mutations are present in a patient sample is central to diagnosis and treatment, because certain mutations cause more severe disease. Schumm et al., in fact, establishes a long-felt need in the art for the present invention, at col. 2:20 – 3:13. Schumm discloses a lengthy list of attempts to multiplex. Schumm also distinguishes STRs from genes causing disease. "While there are multiplex amplification procedures for specific loci, the use of multiplex amplification procedures is greatly desired for the detection of alleles in other types of loci such as STR loci." Col. 3:45 – 48.

14. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and

EXHIBIT A

Curriculum Vitae for Dr. Gary L. Brown

PERSONAL

Name	Dr. Gary L. Brown
Home Address	4 Germantown Ave Christiana, PA, 17509
Place of Birth	Phoenix Arizona
Social Security Number	527-76-0027
Phone	(609) 750-2385 Work (610) 593-5682 Home
E-mail	gbrown@orchid.com
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Work Address	Orchid Biosciences 303 College Road East Princeton, NJ 08540

EDUCATIONAL HISTORY

Ph.D.	University of Oklahoma, 1990, **Molecular Biology** Norman, Oklahoma: DNA-Binding activities and *cis*-acting DNA sequences that Regulate Expression of the *tps* and *ops* genes of *Myxococcus xanthus*
M.S.	Arizona State University, 1985, **Medical Microbiology /Mycology** Tempe, Arizona: Clinical Evaluation of Leathers-Awasthi Medium for the Isolation of *Coccidioides immitis*
B.S.	Arizona State University, 1978, **Microbiology** Tempe, Arizona

PROFESSIONAL EXPERIENCES

Current Assignment

Sr. Intellectual Property Technical Specialist
Orchid Biosciences Sept. 2000 to the present

Prior Assignments

Patent Liaison, Biotechnology
DuPont Agricultural Products
January 1998 to Sept. 2000

Assistant Professor, Mary Washington College,
Fredericksburg Virginia, August 1995 to 1998.

Visiting Assistant Professor, Michigan State University
May 27, 1997-Aug. 15, 1997
May 6. 1996-July 31 1996

Adjunct Professor, Mary Washington College, August 1994-May 1995.

Adjunct Professor, Mary Washington College, Part Time, August 1993- May 1994.

Patent Examiner, Complex Biotechnology (Gene Regulation), U.S. Patent and Trademark Office, Washington, D.C., August 1992 to July 1994.

National Institute of Health Postdoctoral Fellow, John Innes Institute, Norwich, United Kingdom, May 1990 to 1992.

Graduate Research Assistant, University of Oklahoma, Department of Botany and Microbiology, Norman Oklahoma, August 1985 to May 1990.

Prior to 1985

Medical Microbiologist (ASCP Registered)/Technical Director
MicroBio Products, Tempe Arizona

Medical Microbiologist (ASCP Registered), Phoenix Memorial Hospital

Microbiologist (ASCP Registered), Burn Treatment Skin Bank, Phoenix Arizona

HONORS, GRANTS AND AWARDS

Awarded a Scholar in Residence position at Mary Washington College for the 1997-1998 school year. The faculty member is provided a residence on campus and other benefits to foster and promote more campus-wide faculty/student interaction. Faculty candidates are selected on the basis of faculty peer and student recommendations, personal interviews, teaching evaluations and campus community activity.

National Science Foundation Research Opportunity Award (ROA). Identification and Characterization of *S. coelicolor* mutants that suppress the *mia* antibiotic deficient phenotype. Awarded Spring 1996 as a supplement to Dr. Champness existing NSF funding. Research collaboration with Dr. Wendy Champness, Michigan State University, 5/1/96 to 7/31/96.

Mary Washington College Faculty Development Fund: Isolation and characterization of *mia* suppressor genes of *S. coelicolor*. Awarded Spring 1996.

National Institute for Health Postdoctoral Fellowship: "Regulation of the *whiG* gene of *Streptomyces coelicolor*. Fellowship #1F32GM13787-01A1X1, 1991/1992.

John Innes Foundation Fellowship: "*Streptomyces* differentiation", 1990/1991.

PUBLICATIONS

Riggle, P., **G. L. Brown,** B. S. Price and W. Champness. Global transcriptional regulation of antibiotic genes by the *Streptomyces coelicolor mia* locus. Manuscript in preparation.

Kelemen, G., **G. L. Brown**, P. Bryan, K. F. Chater, J. Kormanec, L. Potuckova, M.J. Butner. (1996) The positions of *whiG* and *sigF* in the hierarchy of genes controlling the development of spore chains in the aerial hyphae of *Streptomyces coelicolor A3(2)*. Mol. Micro. 21(3), 593-603.

Chater, K. F., Brian, P., **Brown, G.L.**, Plaskitt, K.A., Soliveri, J., Tan, H., and Vijgenboom,E. (1993). Problems and progress in the interactions between morphological and physiological differentiations in *Streptomyces coelicolor*. In, Industrial Microorganisms: Basic and Applied Molecular Genetics. R.H. Baltz, G.D. Hegeman and P.L. Skatrud, eds. pp. 151-157. American Society for Microbiology, Washington D.C.

Kil, S.-K, **G.L.Brown**, J.D. Downard, 1990. A segment of *Myxococcus xanthus ops* DNA functions as an upstream activation site for *tps* gene transcription. J. Bacteriol.172:3081-3088.

Hartman, R.F., **G.L. Brown**, S.D. Rose, 1981. Methacrylate polymerization by azo RNA: Potential usefulness for chromosomal localization of genes. Biopolymers. 20:2635-2648.

Brown, G.L., R.F. Hartman and S.D. Rose, 1980. Enzymatic addition of modified cytosine nucleotides to DNA;Methacrylate polymerization by an azo pyrimidine. Biophys. Biochem. Acta., 608:266-276.

SELECTED PROFESSIONAL PAPERS (1991-present)

M. Ardolino, B.S. Price, W. Champness and **G. L. Brown**. Sequence analysis of DNA flanking the *mia* locus of *Streptomyces coelicolor*. Presented at the Virginia academy of science annual meeting, May 1997.

J. Ross, W. Champness and **G.L. Brown**. Analysis of *mia* expression in *Streptomyces coelicolor* and *Streptomyces lividans*. Presented at the Virginia Academy of Science annual meeting. May, 1997.

A.L. Charboneau, **G.L. Brown**, and K.E. Loesser. Presented at the Virginia academy of Science Annual Meeting May 1996. Mechanisms of arterial Restenosis in Rabbits: Localization of PDGF-alpha Using *in Situ* Hybridization.

Nowell. K.W., **G.L. Brown** and R. Barra. 1995. Isolation of P53 from ME-180 cells and analysis of the *p53* response to DNA damage induced by treatment with doxorubicin. Virginia Academy of Science Meeting, Richmond Virginia, Virginig Journal of Science Vol. 46, No. 2 1995.

Brown, G.L., K.L. Brown, C.J. Bruton, M.J. Buttner, G. Keleman, H.Tan and K. Chater. 1994. Transcription of *whiG*, a sporulation gene of *Streptomyces coelicolor A3* encoding a putative sigma factor. Annual Meeting of the Virginia Branch American Society for Microbiology, Richmond Virginia.

Brown, G.L., and K. Chater. 1991. Transcriptional Regulation of the *whiG* gene of *Streptomyces coelicolor*. Conference on the Biological Chemistry of RNA Polymerase, Nottingham, England.

INVITED TALKS

G.L. Brown. 1997. Developmental and antibiotic gene regulation in *Streptomyces coelicolor*. Department of Biological Sciences, California Polytechnic State University (Cal Poly) March 12, 1996. Invited by Raul Cano.

G.L. Brown. 1996. Evolution: From Darwin to Mitochondrial Eve. Given to Mary Washington College Bachelor of Liberal Studies students. Invited by Teddesse Adrea.

G.L. Brown. 1995. The position of *whiG* in the hierarchy of genes controlling the development of spore chains in the aerial hyphae of *Streptomyces coelicolor A3(2)*. Research presentation to the laboratory group of Dr. Wendy Champness at Michigan State University.

G.L. Brown. 1994. Transcriptional regulation of *whiG*, a sigma factor gene: Implications for developmental control in *Streptomyces coelicolor*. Lecture given to the Biology department of

Mary Washington College (prior to appointment as Assistant Professor). Invited by Rosemary Barra.

G.L. Brown. 1991. Analysis of the *Streptomyces coelicolor whiG* gene regulatory region. Invited talk at the University of Texas, Department of Microbiology. Invited by Dr. Alan Schauer.

G.L. Brown. 1990. Regulation of *tps* and *ops* gene expression in *Myxococcus xanthus*. Invited talk at the John Innes Institute, Norwich, England. Invited by Keith Chater.

UNDERGRADUATE RESEARCH ADVISEES

Jed Ross	Graduated May 1997 (Research Scientist, Genentech, San Francisco, CA.)
Michele Carr	Graduated May 1997 (Research Scientist, AstraZeneca Wilmington DE)

SUBJECTS TAUGHT

General Biology 121/122

Cell Biology 211

Genetics 341

Biotechnology 471

Molecular Biology 471

EXHIBIT B

CF-12 B Tube Test Development – Monthly Project Report.

Susie Weston - Lab books NBY 5882 and NBY 5922

Previous experiments with CF-12 and alpha-1-antitrypsin had shown that the controls were prone to non-specific priming. Therefore all possible pairwise combinations of the Apo B and KRT 16 control primers were evaluated at 1μM each on normal DNA. All combinations produced a ladder of non-specific bands, some of them pretty strong. The KRT primers appeared particularly non-specific - each produced a strong ladder on its own.
In order to determine whether the CF primers were in general as non-specific as the controls, 4 pairwise combinations of common primers (_F508, 621+1/R117H, R334W and G551D/R553X) were evaluated as above. They were only weakly non-specific.

The direction of the R1162X reaction needed to be reversed in order to avoid picking up the 3617 G/T polymorphism (see Nancy's book for initial experiments done in my absence).
Reversed R1162X 207bp product
Forward - mutant primer, 5' position 172 (Genbank M55126), 30mer
Reverse - common primer, 3' position 349, 30mer
Four CF-12 B mixes, containing the following R1162X primers, were compared.
1)original
2)reversed, R1162XM G-G-2mm (as in the pre-CF12 R1162X single test).
3)reversed, R1162XM G-A-2mm
4)reversed, R1162XM G-G-2mm + C-A-6mm and substituting in _F508N C-T-2mm + G-T-6mm (designed to avoid a primer-dimer between R1162XM and _F508N - see Nancy's book).
Mix 1, although unsuitable, produced a strong, specific R1162X reaction without primer-dimer. Mixes 2 and 4 produced a strong, specific R1162X reaction with strong primer-dimer. Mix 3 produced a weak, specific R1162X reaction with primer-dimer. It was also noticed that larger product bands were weaker than usual, probably due to the inclusion of more inhibitory primers, and there was a weak non-specific R334W with one mouthwash (out of 7 +/+ samples).
Analysis on "Oligo4" revealed a number of primer-dimers involving the reversed R1162X primers. R1162XC had a 4bp 3'overlap with R334WM and R1162XM G-G-2mm had a 3bp 3'overlap with both KRT 16 forward and G551D/R553XC and a 4bp 3'overlap with Apo B forward.
Next, reversed R1162XM G-T-2mm was evaluated (earlier I had mistakenly thought this primer wasn't an option). Analysis on "Oligo4" had shown that this could form a 4bp 3'overlap with 621+1/R117HC, but this wouldn't be a difficult fix. It produced a strong R1162X reaction and was only more non-specific than reversed R1162XM G-G-2mm with one sample. As it was involved in fewer primer-dimers, reversed R1162XM G-T-2mm was introduced as standard.

KodakTaq was introduced as standard.
A new reversed R1162XC was designed to avoid the primer-dimer with R334WM. This primer was evaluated in a mix containing as many purified syntheses as possible to reduce the zinc content to a minimum. It appeared to work as well as the first reversed R1162XC and was therefore introduced as standard:
R1162X 200bp product

Forward - mutant primer, 5' position 172 (Genbank M55126), 30mer
Reverse - common primer, 3' position 343, 29mer
The use of a "low zinc" mix increased band intensities, although R553X remained rather weak. 3 (out of 8) +/+ samples gave weak non-specific R1162X bands, 1 also gave a weak non-specific R334W band. There was _F508 normal contamination in the 2 -ve controls, possibly due to contamination of a purified primer (Stephen also noticed this when he first evaluated the purified CF-12 B primers). Also, there was still some primer-dimer, probably due to the 4bp 3'overlap between R1162XM and 621+1/R117HC and/or the 3bp 3'overlap between R334WM and _F508N.

INITIAL CHALLENGE STUDIES

Although not perfect, CF-12 B was put through an initial challenge in order to get a feel for its performance.
A large mix was prepared using purified primers where possible (from Cellmark: 9 purified primers, 1 crude "inhibitory" 60mer, 1 crude "zinc free" primer; from Oswel: 5 crude primers).
As with CF-12 A, 50ng DNA per reaction was introduced as standard and the following DNA panel was used: 621+1/_F, R553X/+, G551D/_F, R117H/+, R1162X/+, R334W/+, _F/+, 2x_F/_F, K562+/+, 2xalk+/+ and 2xHLA+/+.

ANNEALING TEMP. +/- 2°C

56°C - weak 621+1 and R553X
- specific
- strong primer-dimer)see previous
- _FN contamination in -ve)expt.

58°C - weak 621+1 and R553X
- weak non-specific R1162X with all _F/_F and +/+ DNAs
- primer-dimer
- _FN contamination in -ve

60°C - very weak 621+1 and R553X, weak G551D and R117H, weaker _FN, stronger upper control
- possibly non-specific R1162X with one _F/_F and _FN with other _F/_F
- weak primer-dimer
- _FN contamination in -ve

TAQ +/- 20%

(primer-dimer throughout and _FN contamination in -ve as above)

1.6U - very weak 621+1 and R553X
- specific

2.0U - weak 621+1 and R553X
- possibly some non-specific R1162Xs

2.4U - weak R553X
- weak non-specific R1162X with some _F/_F and +/+ DNAs

2 MIN.S ANNEALING +/- 2°C

Aim: to increase weak diagnostic band intensities
(primer-dimer throughout and _FN contamination in -ve as above)

56°C - weak 621+1 and R553X
- specific

58°C - weak R553X
- weak non-specific R1162X with all _F/_F and +/+ DNAs

60°C - weak 621+1 and R553X
- weak non-specific R1162X with all _F/_F and +/+ DNAs and some mutant DNAs

2 minute annealing was introduced as standard

As mentioned previously there was a 4bp 3'overlap between R1162XM and 621+1/R117HC and this was probably responsible for the primer-dimer seen on gels. In order to eliminate this a new 621+1/R117HC was designed as follows:
621+1/R117HC C-T-8mm T-C-26mm G-A-27mm
5' position 119 (Genbank M55109, M55500, M55501), 34mer
This was evaluated alongside a couple of subtle changes to the basic mix. Also, all of the crude CF-12 B primers used here were for the first time "zinc free" - 14 from Cellmark, 2 from Oswel.
Mix 1 - original
Mix 2 - new 621+1/R117HC - designed to avoid primer-dimer
Mix 3 - mix 2 plus 0.5 x concentration of R334WC, R1162XM and R1162XC in an attempt to eliminate non-specifics
Mix 4 - mix 3 plus 2 x concentration of 621+1M and R553XM in an attempt to increase these diagnostics
The results from mixes 2 and 3 were inconclusive as mix 1 failed to generate its usual strong primer-dimer band and gave only a few weak non-specifics. Mix 4 resulted in stronger 621+1 and R553X bands. Taking into account that the changes in mixes 2 and 3 had no adverse effect and were potentially beneficial the new 621+1/R117HC, 0.5µM R334WC, R1162XM and R1162WC and 2µM 621+1M and R553XM were introduced as standard.

INITIAL CHALLENGE STUDIES cont'd
Using the primers and format as described above a large mix was prepared for further challenge studies. It was QC'd using the standard DNA panel at both 1 and 2 min. annealing. With 1 min. the larger diagnostics were rather weak and with 2 min. the diagnostics looked fine although both _F/_F DNAs showed non-specific R1162X and R334W bands and all +/+ DNAs showed non-specific R1162X bands. 2 min. annealing was kept as standard.
50, 10, 5, 1, 0.5, 0.1ng DNA per reaction
(used all except the alk and HLA DNAs from the standard panel)
50, 10, 5ng - OK
1, 0.5, 0.1ng - faint, smeared
0.1ng - 621+1, R117H, R1162X, R334W and _FN present
- R553X absent (upper control also absent)
- G551D absent (but upper control present)
Also, both _F/_F DNAs showed weak non-specific R1162X and R334W bands down to 1/0.5ng and one possibly showed non-specific _FN bands.
50, 100, 200, 300, 400, 500ng DNA per reaction
(used 2x_F/_F, 2x_F/+, K562+/+ and 2xHLA+/+ DNAs)
There was no increase in any band intensities (specific or non-specific) with an increasing amount of template. Both _F/_F DNAs showed weak non-specific R1162X and R334W bands, K562 showed weak non-specific R1162X bands, one _F/+ possibly showed non-specific R1162X bands and the other _F/+ and +/+ DNAs were specific.

EXHIBIT C

CF-12 B Tube Test Development – Monthly Project Report

Susie Weston - Lab books NBY 5922 and NBY 5935

Following Initial Challenge Studies further formulations of the multiplex were evaluated in order to try and improve its performance.

The aim of the first experiment was to eliminate non-specific R1162X and R334W bands and increase the band intensities from the "overARMS" reactions. 4 mixes were compared:
Mix 1 - standard
Mix 2 - 0.25μM each R1162X, 0.25μM each R334W
Mix 3 - 0.1μM each R1162X, 0.1μM each R334W
Mix 4 - mix 2 plus 1.5μM exon 4C, 1.5μM exon 11C
0.25μM R1162X and R334W primers appeared to eliminate the non-specifics and didn't affect the diagnostics. At 0.1μM the diagnostics were too weak. 1.5μM exon 4C and exon 11C primers possibly increased the diagnostics. 0.25μM each R1162X, 0.25μM each R334W, 1.5μM exon 4C and 1.5μM exon 11C were introduced as standard.

The aim of the second experiment was to balance _F508N and diagnostics better and further decrease the possibility of non-specifics (particularly with _F/_F DNA). 4 mixes were compared:
Mix 1 - standard
Mix 2 - 0.75μM each _F508
Mix 3 - 0.3μM each KRT
Mix 4 - mix 2 plus mix 3
The results from all mixes were almost identical but as mix 4 had the greatest potential to balance band intensities and eliminate non-specifics, 0.75μM each _F508 and 0.3μM each KRT were introduced as standard.

It was thought that as the ApoB primers had higher Tms than the diagnostics (forward: 29mer, Tm 75.1°C; reverse: 30mer, Tm 75.3°C) false negatives might occur at high annealing temperatures. Therefore they were shortened at the 5'end as follows:
Forward: 24mer, Tm 69.1°C
Reverse: 26mer, Tm 68.9°C
New product size: 491bp
The shorter primers were substituted into the multiplex at the same concentration as the originals and produced a band of equal intensity. Therefore the shorter ApoB primers were introduced as standard.

In an attempt to further decrease the possibility of non-specific R1162X bands with _F/_F DNA the R1162XC primer was shortened (original: 29mer, Tm 70.0°C; new: 25mer, Tm 65.5°C) in the hope that it would prime less efficiently. This surpisingly had the reverse effect - all _F/_F DNAs were specific with the original primer and non-specific with the new primer.

In order to evaluate the effect of the _F508N reaction on diagnostic intensities and to see if R1162XM A-G-2mm could produce a strong, specific reaction the following mixes were compared:
Mix 1 - standard
Mix 2 - no _F508 primers
Mix 3 - 0.5µM each _F508
Mix 4 - R1162XM A-G-2mm, 2µM each R1162X (evaluated before, but in an "inhibitory" mix)
Dropping the _F508N reaction from the multiplex increased diagnostic intensities. 0.5µM _F508 primers didn't significantly affect band intensities, although the _F508N and diagnostic reactions were possibly better balanced. R1162XM A-G-2mm resulted in a very weak, specific R1162X reaction. 0.5µM each F508 was introduced as standard.

CF-12 B, incorporating the improvements made (1395-01.P4), was put through Developmental Challenge Studies and performed as follows. The DNA panel used was the same as in the last challenge studies (see report dated 24.8.95) except for annealing and Taq challenge where R553X/_F and R553X/G551D DNAs were also used and the alk. DNAs were dropped.
ANNEALING TEMP. +/- 2°C
56°C - OK
58°C - weak non-specific R1162X and R334W with _F/_F DNAs
60°C - weak 621+1
Actions taken : 1) All _F/_F reactions run side by side on same gel. Weak non-specific R1162X and R334W bands at both 58°C and 60°C.
2) 2 621+1 DNAs amplified in various block positions at 60°C. 621+1 bands slightly weak but OK.
TAQ +/- 20%
1.6U - very weak R553X (although R553X/G551D OK)
2.0U - OK
2.4U - CF12-B at its best
DNA CONCENTRATION - 50, 10, 5, 1, 0.5, 0.1ng/reaction
50, 10, 5ng - OK
1, 0.5ng - all diagnostics present but weak to very weak
0.1ng - only 621+1 and R1162X present, possible false negative with G551D/_F DNA
DNA CONCENTRATION - 50, 100, 200, 300, 400, 500ng DNA/reaction
No increase in any band intensities, specific or non-specific, with an increasing amount of template. Both _F/_F DNAs specific, although the odd weak non-specific R1162X band with 2 out of 3 +/+ DNAs.

CF-12 B Tube Test Development –Monthly Project Report

Susie Weston - Lab books NBY 5935 and NBY 6107

The last set of Developmental Challenge Experiments (on 1395-01.P4) indicated weaknesses in the test and various experiments were undertaken this month in order to try and improve things.

621+1 and R553X tended to be the weakest of the diagnostics and an increasing dNTP concentration was evaluated in an attempt to increase band intensities. At 100 (standard) and 125μM dNTP band intensities were similar but at 150 and 200μM dNTP band intensities (particularly larger product bands) decreased. The results suggested that at 150μM dNTP and above there was not enough free magnesium for the Taq to function optimally - dNTPs directly chelate a proportional number of magnesium ions and therefore increasing [dNTP] reduces [free magnesium]. The results also suggested that under standard conditions the larger product reactions just reach the plateau phase whereas smaller product reactions get there much earlier.

Previous experiments on both CF-12 A and B had indicated that the KRT 16 primers were particularly prone to mispriming. Stephen designed 5 new pairs of lower control primers and his initial experiments showed that the following 4 pairs were suitable for further evaluation: ODC(1), ODC(2), CAX-3 and IL-2. All 4 pairs were substituted individually into CF-12 B at the standard concentration and compared to KRT 16. ODC(1) possibly weakened the diagnostics. CAX-3 and IL-2, although they produced strong control bands, reduced the specificity of the test. IL-2 also resulted in primer-dimer in the negative. ODC(2) produced a weaker control band but as the diagnostic intensities and specificity appeared identical to the mix with KRT 16 it appeared most promising.

The following R1162X mutant primers were evaluated in mixes with and without the KRT control in an attempt to increase the specificity of the reaction:
1) 30mer, T-G-2mm (standard)
2) 30mer, T-G-2, C-A-6mm
3) 25mer, T-G-2mm
4) 25mer, T-G-2, C-A-6mm
All primers were specific with the KRT control in the mix but non-specifics could be seen clearly with _F/_F DNAs and the KRT control absent. Primers 1) and 3) gave similar results - both diagnostics and non-specifics were strongest. Primer 2) gave weak diagnostics and weak non-specifics and primer 4) gave very weak diagnostics and extremely weak non-specifics. Primer 1) was kept as standard.
In the same experiment a 621+2T>C DNA was evaluated on CF-12 B. The sample supplied was labelled "dilute" and was therefore evaluated at 1 in 20 and 1 in 2. It was thought it might come up 621+1 as only the 3'base would be mismatched but much to our relief it didn't.

After analysis of both Stephen and my results in which the new lower control primers were evaluated it was decided to introduce the ODC(2) primers as standard.
ODC 97bp product
Forward - "bubble" primer, 5'position 1312 (Genbank M34158), 30mer, C-T-2mm
Reverse - "bubble" primer, 3'position 1379, 30mer, A-C-2mm

A decreasing Apo B upper control primer concentration was evaluated in an attempt to further weaken the reaction and avoid the possibility of false negatives (as seen in the challenge with a G551D/_F DNA). At 0.075μM each Apo B primer the band was a little weaker than at 0.1μM (standard) and at 0.05μM the band was too weak. At the same time a G551D/+ DNA supplied for the Abingdon CF(4)M QC panel was evaluated as a possible challenge sample at 50 to 0.1ng/reaction . It amplified quite badly - either it was a poor sample or the DNA concentration

measurement (done on the Abingdon fluoroskan) was incorrect. 0.075µM each Apo B primer was introduced as standard.

CF-12 B, incorporating the improvements made (3435-04.P5), was put through Developmental Challenge Studies and performed as follows. The mix was QC'd using a panel of 10 phenol prep. blood DNAs and at the same time the 621+2T>C and 3617G/T DNAs were re-checked on the test. The R117H/+ DNA almost failed but the upper control dropped out first (and was OK when repeated). The 621+2 and 3617 DNAs didn't come up 621+1 and R1162X respectively.
DNA CONCENTRATION - 50, 100, 200, 300, 400, 500ng DNA/reaction

* Experiment performed with 2x_F/_F, 1x_F/+, K562+/+ and 2xHLA+/+ phenol prep. DNAs using same gel conditions and camera settings as previous.
Band intensities did not increase with an increasing amount of template. There were no non-specifics.

At this point J+J decided that CF-12 should be developed for alk. prep. blood DNA and therefore no further prototype 5 challenge experiments were performed using phenol prep. blood DNA at 50ng/reaction as standard.

Following CF-12 evaluation by Nikki D.'s group (using alk. prep. blood validation DNAs) it was noticed that band intensities at Abingdon tended to be stronger and although CF-12 A appeared specific at Gadbrook it was clearly non-specific at Abingdon. During a visit to Abingdon various experiments were undertaken in order to determine the cause.
Expt. 1) An Abingdon 480 was monitored with the Gadbrook Squirrel. Results not yet analysed.
Expt. 2) PCR set up in parallel with Paul. 4 validation DNAs on A mix prototype 3 (KRT control), same 4 DNAs on A mix prototype 4 (ODC control), 4 phenol prep. DNAs on B mix prototype 4. Gels run and photographed by both the Abingdon and the Gadbrook standard methods.

Abingdon standard: 0.1µg/ml EtBr
20µl PCR product + 10µl gel loading buffer
shutter setting 2

Gadbrook standard: 0.5µg/ml EtBr
25µl PCR product + 10µl gel loading buffer
shutter setting 8

For PCR set up, Abingdon and Gadbrook operator and method differences were insignificant and did not affect the end result. The combination of 0.1µg/ml EtBr and shutter setting 2 made A mix non-specifics most visible. Decreasing [EtBr] made the background darker. Standard Gadbrook conditions didn't reveal any non-specifics. Substitution of the ODC control into the A mix considerably reduced non-specifics (analysis on the UVP system confirmed this result).
Expt. 3) Doubling dilutions of Phi-X-174 HaeIII from 1µg/20µl were run across a 0.1 EtBr gel. At shutter setting 2 bands were visible down to 1ng and at shutter setting 8 bands were visible down to 15ng. This was later repeated at Gadbrook and equivalent results were obtained if the screen over the transilluminator was removed. With the screen in place the background was lighter and visibility was slightly reduced.
Expt. 4) CF-12 B products from Gadbrook were analysed at Abingdon under Gadbrook conditions. The results looked similar to those obtained at Gadbrook except the background was darker.
All gels were re-photographed at Gadbrook approx. 6hrs later. The backgrounds appeared lighter and non-specifics couldn't be seen, possibly due to diffusion.

"Pack leaflet" conditions (0.1µg/ml EtBr, 25µl PCR product + 10µl glb) and shutter setting 2 were introduced as standard.

The following bloods were alk. prep.'d for CF-12 challenge: 6x +/+ from Pharms, 1x suspected R553X/+, 1x 1717-1/+ (as Abingdon prep. poor) and 1x _F/_F (done four times). All were checked on CF-12 and were fine except the R553X band was weak. Nikki D. quantitated the samples on the fluoroskan and they ranged from 27 to 53ng/5µl.

Developmental Challenge Studies on CF-12 B prototype 5 were continued as follows. The DNA panel consisted of 621+1/+, R553X/+, G551D/+, R117H/_F, R1162X/_F and _F/+ at 16 to 44ng/5µl alk. prep.'d at Abingdon, 2x +/+ and 2x _F/_F alk. prep.'d at Gadbrook and R117H/+, R1162X/+ and R334W/+ phenol prep.s at 50ng/5µl.

TAQ +/- 20%
1.6U - R553X and G551D weak
2.0U - OK
2.4U - CF-12 B at its best

ANNEALING TEMP. +/- 2°C
56°C - OK, R553X and G551D weakest
58°C - OK, R553X and G551D weakest
60°C - 621+1, R553X, G551D and R117H (alk. prep. only) weak

DNA CONCENTRATION - stock, 10, 5, 1, 0.5, 0.1ng/reaction
stock, 10, 5, 1ng - OK, R553X weakest
5, 1, 0.5, 0.1ng - the odd non-specific below lower control
0.5, 0.1ng - 621+1, G551D, R117H and R1162X DNAs: various very weak non-specific diagnostics (probably due to weaker lower control at low [DNA])
0.1ng - All diagnostics except R334W present. 2 DNAs: upper control drop-out, 2 DNAs: lower control drop-out

the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Gary L. Brown

Dr. Gary L. Brown

1-22-2002

Date